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Biotransformation of Butyltin Compounds Using Pure Strains of Microorganisms

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The debutylation of tributyltin chloride by several strains of fungi, yeasts and bacteria is described. Under standard conditions and with low initial concentration of substrate, significant biotic degradation of tributyltin (6–32%) was detected after five days at 28 °C. Dibutyltin and monobutyltin were formed in all cases,with higher yields of the latter. Two microorganisms catalysed the transformation of monobutyltin to dimethyltin and trimethytin whereas all microorganisms were able to methylate inorganic tin(IV) to trimethyltin. Our results suggest that tributyltin biodegradation by microorganisms is generally possible, provided sufficiently low concentrations of substrate are used.

Keywords: biodegradation; debutylation; biomethylation; tributyltin; dibutyltin; monobutyltin; tin(IV); microorganisms; pure strains

INTRODUCTION

The presence of organotin compounds in the marine environment arose primarily from the use of antifouling agents in marine paints. The toxic component of such agents is a tributyltin (TBT) derivative, i.e. the oxide (TBTO) or fluoride (TBTF). This compound, with two degradation products, namely dibutyltin (DBT) and monobutyltin (MBT) species, is found in sea and lagoon waters[1] and in sediments,[1–6] as well as in aquatic organisms.[7–12] In France, the use of tributyltins has been regulated since 1982.

During the last ten years there have been several reports on the ability of microorganisms isolated from seawater or sediments, or of pure strains of microorganisms, to biotransform organotin compounds or inorganic salts of tin(II) or tin(IV).[13] Table 1 shows the conditions used in the various studies concerned with TBT, DBT and MBT. Initial concentrations of TBT as its bis(tributyl) oxide vary to a large extent (between 1 $\mu g\,l^{-1}$ to 2.5 $mg\,l^{-1}$), as do the incubation times (2–48 weeks). Such diversity of conditions precludes any general comparison of the efficiency of the biotic degradation of TBT and its metabolites. Moreover, the high concentrations of substrates often used may have adverse effects on development of the microorganisms, thus preventing or limiting degradation. Having at our disposal an efficient and sensitive analytical method for organotin compounds, we therefore decided to re-examine the biodegradation of TBT, DBT and MBT compounds in the presence of well-defined pure strains of microorganisms, under strictly controlled conditions. In the present work, we report the degradation of tributyltin chloride (TBTCl), dibutyltin chloride ($DBTCl_2$) and monobutyltin chloride ($MBTCl_3$) in the presence of several bacterial yeast and fungal strains. The production of methyltin derivatives from inorganic tin(IV) under the same conditions has also been examined. Particular attention has been given to differentiate biotic and abiotic conversions, and to identify all metabolites formed.

MATERIALS AND METHODS

Organotin compounds were purchased from Merck (TBTCl, $DBTCl_2$ and $MBTCl_3$), inorganic salts from Prolabo and culture medium nutrients from Fluka.

Sterile conditions were maintained during the operations of handling the microorganisms. Seven microorganisms (fungi, yeast and bacteria) available from our collection were selected on the bases of their ability to develop in the presence of

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Table 1 Various selected conditions used to biotransform TBT in previous studies

Source of microorganisms	Nutrient	Incubation time (days)	Temp. (°C)	Aeration	Initial tin concn ($mg\,l^{-1}$)	Products (%)	Ref.
Sediments	Yes	26	20	Yes	1 (in TBTO)	TBT(46), DBT(0.4), MBT(0.4), Sn^{4+}(2.4)	14
Sediments	No	26	20	Yes	1 (in TBTO)	TBT(29), DBT(8), MBT(2), Sn^{4+}(6)	14
Sediments	Yes	26	20	No	1 (in TBTO)	TBT(52), DBT(5), MBT(4), Sn^{4+}(17)	14
Sediments	No	26	20	No	1 (in TBTO)	TBT(17), DBT(6), MBT(2), Sn^{4+}(63)	14
Seawater	No	14	22	Yes	0.1 (in TBT)	No degradation	16
Seawater	No	14	5	Yes	0.001 (in TBT)	Weak degradation to DBT	16
Seawater	No	14	28	Yes	0.001 (in TBT)	Weak degradation to DBT, MBT	16
Natural water	No	336	20	Yes	1 (in TBT)	TBT(50), DBT(12), MBT(18)	17
P. aeruginosae	Yes	30	30	Yes	2.5 (in TBTO)	TBT(10), DBT(1), MBT(75)	15
Coniophora puteana	Yes	30	30	Yes	1 (in TBTO)	TBT(1), DBT(2), MBT(14)	15
Trametes versicolor	Yes	30	30	Yes	1 (in TBTO)	MBT(50)	15

controlled amounts of organotin compounds: *Chaetomium globosum* (NRRL 1870), *Penicillium citrinum* (NRRL 1843), *Aspergillus tamarii* (NRRL 427), *Schizosaccharomyces pombe* (NRRL Y-9), *Saccharomyces cerevisiae* (NRRL Y-129), *Saccharomyces cerevisiae* (NRRL Y-409) and *Pseudomonas fluorescens* (NRRL B-10). All microorganisms were from Northern Regional Laboratories, Agricultural Research Services, Peoria, Illinois, USA.

Lyophilisates of microorganisms were suspended in sterile water for 24 h and the resulting suspension used to inoculate the appropriate culture medium: beef extract, peptone and dextrose for bacteria; yeast extract, malt extract, peptone, dextrose in phosphate buffer (pH 6.5) for yeast; malt extract, peptone and dextrose for fungi.[18] Maintenance of cultures was carried out on agar slants which were stored for a maximum of six months at 5 °C. For each microorganism, the maximum level of TBTCl acceptable without affecting growth was determined by adding increasing amounts of the organotin compound ($0–3\,ng\,l^{-1}$) to six culture flasks containing approximately equal populations of that microorganism in the corresponding medium.

A sample of microorganism culture was used to inoculate 50 ml of the appropriate medium contained in a 250 ml culture flask. After 48 h at 28 °C in a longitudinal shaker under aerobic conditions, 5% of the suspension (stage I cultures) was used to inoculate 50 ml of fresh medium in a 250 ml flask (stage II cultures). After an 8 h agitation period, stage II cultures received the organotin substrates dissolved in methanol ($50\,\mu l$ of $10\,mg\,l^{-1}$ solution: initial concentration corresponding to $10\,\mu g\,l^{-1}$) or $SnCl_4$ dissolved in concentrated sulphuric acid ($50\,\mu l$ of $20\,mg\,l^{-1}$ solution: initial concentration corresponding to $20\,\mu g\,l^{-1}$). The cultures were then shaken at 28 °C for five days (120 rpm) and samples were taken for analysis from the incubation media.

The biomass was filtered (fungi) or separated from the liquid phase by centrifugation (yeasts and bacteria). Fractions of 1–5 ml of liquid phase were acidified with acetic acid and analysed immediately. Organotin compounds were extracted from the biomass by stirring in acetic acid for 12 h and centrifugation.[19]

Quantitative analysis of the organotin compounds was accomplished by the method previously developed in our laboratory and described in earlier publications.[19–21] It involves several steps: hydride generation, cryogenic trapping, gas chromatography and atomic absorption spectroscopy. Reproductibility of the method was 4–6% with lower limits of detection of $0.2–0.3\,ng\,l^{-1}$ for methyltins and monobutyltin, $0.4\,ng\,l^{-1}$ for di-

butyltin and 0.6 ng l^{-1} for tributyltin.[20] Since the original method involved numerous titrations, the standard addition procedure was not used. Instead, standardization regression curves obtained from measurements in each culture medium were used for subsequent titrations. This procedure is not valid for inorganic tin(IV) titration since the sodium borohydride used for hydride generation contains traces of this cation. Therefore tin(IV) recovery cannot be calculated.

The experimental results are expressed as the average of two or three measurements. In all cases, the standard deviation for each result was less than 10%.

RESULTS AND DISCUSSION

Prior to studying the biotransformation of butyltins, it was necessary to determine the toxicity levels affecting the growth of the microorganisms to be used. Since it is known that tributyltin is generally the most toxic of the organotin compounds employed in this study, we limited our study to TBTCl. The maximum concentrations of organotins compatible with microorganism growth are reported in Table 2. For this determination, we used only a visual comparison: for each strain, Erlenmeyer flasks were incubated with an increasing amount of TBTCl by successive approaches (0–3000 μl^{-1} following the strain studied). Five days later, the concentration corresponding to a fairly inhibited growth was considered as the upper limit compatible with microorganism growth. In most cases, the upper limit was in the range of 10–50 $\mu g\, l^{-1}$, consistently with the published organotin toxicity range of 1–50 ppb.[13] *Saccharomyces cerevisiae* strains, however, seem to tolerate much higher concentrations of TBTCl. It should be noted that previously reported experiments involving *Sacch. cerevisiae*, known to induce methylation, have employed concentrations of various tin species as high as 2 g l^{-1}.[22]

Table 2 Toxicity of tributyltin chloride with respect to the microorganisms studied

Microorganism	Maximum concentration of TBTCl compatible with microorganism growth ($\mu g\, l^{-1}$)
C. globosum	20
P. citrinum	50
A. tamarii	50
Sch. pombe	50
Sacch. cerevisiae	2000
Sacch cerevisiae	2000
Ps. fluorescens	10

From the results reported in Table 2, we selected a value for the initial concentration of butyltins of 0.01 mg l^{-1}, for all the microorganisms. This value is about one order of magnitude higher than the concentrations of organotins found in the environment but two orders of magnitude less than the concentrations used in previously reported bioconversion studies.[14–17]

For each organotin compound, the experimental procedure involved the following steps: determination of amounts of substrate and degradation products in the liquid phase; similar analysis of the isolated biomass; and measurement of the abiotic degradation of the organotin in the corresponding culture medium without microorganisms.

Biotransformation of tributyltin chloride

The data reported in Tables 3 and 4 have been reduced to percentages of tin in all cases. As expected, TBTCl has undergone abiotic degradation in all three culture media in the absence of microorganisms under the same conditions as in the bioconversion experiments. Chief degradation products were DBT (1–7% of the initial TBT) and MBT (3–13%), with recovery ratios varying from 95 to 115%.

The results of TBTCl degradation experiments in the presence of microorganisms (Table 3) show that, in all cases, DBT and MBT were obtained either in solution or from the biomass, or both. Moreover, in most experiments MBT appears as the major degradation product. The high recoveries (in the range of $100 \pm 10\%$) suggest that only very small amounts of inorganic tin have been formed. Taking into account the non-enzymic conversion allows the calculation of the overall proportion of enzymically transformed TBTCl (Table 3). It is clear that all seven microorganisms have the ability to metabolize TBTCl to various extents ranging from 6 to 32%. However, the partition of the substrate remaining at the end of the reaction, and the resulting metabolites (DBT and MBT), between the solution and the biomass varies widely with the nature of the organotin and the particular microorganism (Table 4). For fungi, TBT and DBT were mainly recovered from

Table 3 Biotransformation of tributyltin chloride: recovery of substrate and products (% of tin)

Microorganism	TBT		DBT		MBT		Tin recovery	TBT recovery		Biodegradation (%)
	A[a]	B[a]	A	B	A	B		Incubations	Abiotic tests	
C. globosum	84	0	14	0	5	26	129	84	90	6
P. citrinum	58	0	16	2	4	17	97	58	90	32
A. tamarii	67	0	24	0	7	10	108	67	90	23
Sch. pombe	30	35	2	6	0	35	108	65	86	21
Sacch. cerevisiae	60	12	3	1	0	11	87	72	86	14
Sacch. cerevisiae	42	14	4	19	0	20	99	56	86	30
Ps. fluorescens	23	53	0	0	1	18	95	76	92	16

[a] A, biomass; B, solution.

the biomass, whereas MBT was mostly in solution. For yeasts and bacteria, there was no apparent trend, except that MBT was recovered almost exclusively from solution. It has not been determined whether the organotins associated with the biomass were adsorbed on the external part of the cells or included within them. It seems likely that the partition of butyltins between solution and biomass is governed, at least in part, by their relative hydrophobic characteristics (cf. the large proportion of monobutyltin in solution).

Biotransformation of dibuytyltin chloride and monobutyltin chloride

The same approach was used to study the biodegradation of $DBTCl_2$ and $MBTCl_3$ in the presence of the same set of microorganisms.

For the biodegradation of $DBTCl_2$, the results are reported in Tables 5 and 6. From $DBTCl_2$, the only metabolite detected in all cases was MBT. The overall bioconversion of DBT to MBT was especially important for fungi, where it amounted 10–26%. Generally, however, most of the degradation was abiotic. For dibutyltin degradation, no general rule can be deduced from butyltin partitions between biomass and solution. These results are different from the partitioning results shown in Table 4. This difference can be explained by invocation of a large non-enzymic degradation for the DBT process which induces other pathways of metabolism.

For $MBTCl_3$ biodegradation, in biotic conditions, only *Ps. fluorescens* and *Schiz. pombe* showed the ability to biotransform MBT. In both cases, the only degradation products detected were methyltins. From *Ps. fluorescens*, a 10% degradation of MBT was observed with an apparently quantitative transformation into dimethyltin, which was found exclusively in solution. Under the same conditions, *Schiz. pombe* metabolized 13% of MBT, and corresponding quantities of dimethyltin (DMT) (10%) and trimethyltin (TMT) (3%) were produced. In both experiments, we were unable to detect inorganic tin(IV), which is the probable intermediate in the formation of methyltins from MBT. It is worthwhile to note that all these yields of biodegradation are quite high in comparison with previous

Table 4 Partition of butyltin compounds between biomass and solution in TBT biodegradation

Microorganism	TBT (%)		DBT (%)		MBT (%)	
	Biomass	Solution	Biomass	Solution	Biomass	Solution
C. globosum	100	0	100	0	16	84
P. citrinum	100	0	89	11	19	81
A. tamarii	100	0	100	0	42	58
Sch. pombe	46	54	25	75	0	100
Sacch. cerevisiae	83	17	75	25	0	100
Sacch. cerevisiae	75	25	17	83	0	100
Ps. fluorescens	30	70	0	0	5	95

Table 5 Biotransformation of dibutyltin chloride: recovery of substrate and products (% of tin)

Microorganism	DBT		MBT		Tin recovery	DBT recovery Incubations	Abiotic tests	Biodegradation (%)
	A[a]	B[a]	A	B				
C. globosum	15	50	10	27	102	23	33	10
P. citrinum	18	23	0	96	137	18	33	15
A. tamarii	3	62	4	50	119	7	33	26
Sch. pombe	11	9	0	70	90	12	10	0
Sacch. cerevisiae	5	2	8	75	90	13	10	0
Sacch. cerevisiae	15	9	3	75	122	18	10	0
Ps. fluorescens	55	74	3	18	150	55	61	6

[a] A, biomass; B, solution.

studies in which the incubation times were not less than six times longer.[14–17]

Biotransformation of inorganic tin(IV)

As in the case of MBT, no transformation of inorganic tin(IV) was observed in the culture media after a five-day incubation period in the absence of microorganisms. In contrast, all microorganisms studied induced biomethylation of inorganic tin(IV) in small proportions under the same conditions. The only product detected in these biotransformations was TMT, and it was found essentially in solution (Table 7). The largest bioconversion ratios were observed with yeast. The results of Table 7 suggest that the inability of five microorganisms (exceptions: *Schiz. pombe* and *Ps. fluorescens*) to catalyse the transformation of monobutyltin to methyltin is primarily due to the failure of MBT to undergo enzymic degradation rather than the inability of the microorganisms to methylate inorganic tin(IV) under these conditions. These results have to be referred to some previous studies,[23–28] giving methyltins with natural sediments, waters or co-cultures. But a true comparison is unrealistic because of different media and unidentified microorganisms in most of these studies.

Table 6 Partition of butyltin compounds between biomass and solution in DBT biodegradation

Microorganism	DBT (%)		MBT (%)	
	Biomass	Solution	Biomass	Solution
C. globosum	57	43	66	34
P. citrinum	100	0	19	81
A. tamarii	42	58	55	45
Sch. pombe	95	5	80	20
Sacch. cerevisiae	100	0	12	88
Sacch. cerevisiae	40	60	3	97
Ps. fluorescens	83	17	8	92

Table 7 Bioconversion of inorganic tin(IV)

Microorganism	TMT (ng)		Biotransformed tin (%)
	A[a]	B[a]	
C. globosum	0	22	2
P. citrinum	0	13	1
A. tamarii	0	22	2
Sch. pombe	5	63	7
Sacch. cerevisiae	12	137	15
Sacch. cerevisiae	10	145	16
Ps. fluorescens	0	55	6

[a] A, biomass; B, solution.

CONCLUSION

Our results confirm the conclusion of a preceding study on metabolism of TBT by pure strains of a small set of microorganisms.[15] However, since our incubation conditions were closer to environmental conditions (especially the initial concentration of TBT), our results indicate that enzymic degradation of TBT is one of the major causes of TBT instability in the environment. Biotic degradation of TBT, defined as the difference between overall degradation and abiotic degradation in sterile culture media, was significant after a five-day incubation period at 28 °C for all of the microorganisms studied. Taking account the effect of chemical alteration of the medium on microorganism growth, we also recently conducted similar experiments with resting cells and butyltins as the sole carbon source in a buffer.

The results of these experiments corroborate those obtained in the present work and emphasize the importance of enzymic degradation of TBT (O. Errécalde, M. Astruc, G. Maury and R. Pinel, unpublished results).

With all microorganisms, the major product of TBT degradation was MBT, which is less toxic and more soluble than the tributyl derivative. The accumulation of MBT seems to be the result of DBT instability in sterile culture media[15] as well as the relative stability of MBT with respect to enzymic degradation. Two microorganisms, however, were able to transform MBT to methyltins with good yields (10 and 13%). This is the first example of the direct production of methyltins from monobutyltin using pure strains of microorganisms. It seems likely that inorganic tin(IV) is an intermediate in this reaction since biomethylation of inorganic tin is a well-documented phenomenon. However, since tin(IV) cannot be detected in these experiments, an alternative process might be operative, namely the methylation of MBT followed by debutylation. Tributylmethyltin and dibutylmethyltin have been found in sediments,[29] suggesting that butyltin species can be methylated. No mixed methylbutyltin compounds were found in our experiments, and thus the first type of mechanism seems more plausible. Experiments currently in progress involving resting cell systems should provide additional information concerning the biotransformation of TBT and its metabolites.

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